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Dynamics of extended-spectrum cephalosporin resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec, Canada

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ABSTRACT

The aim of this study was to investigate the evolution with time of ceftiofur-resistant *Escherichia coli* clinical isolates from pigs in Québec, Canada, between 1997 and 2012 with respect to pathotypes, clones and antimicrobial resistance. Eighty-five ceftiofur-resistant *E. coli* isolates were obtained from the OIE (World Organisation for Animal Health) Reference Laboratory for *Escherichia coli*. The most prevalent pathovirotypes were enterotoxigenic *E. coli* (ETEC):F4 (40%), extraintestinal pathogenic *E. coli* (ExPEC) (16.5%) and Shiga toxin-producing *E. coli* (STEC):F18 (8.2%). Susceptibility testing to 15 antimicrobial agents revealed a high prevalence of resistance to 13 antimicrobials, with all isolates being multidrug-resistant. bla_{CMY-2} (96.5%) was the most frequently detected β-lactamase gene, followed by bla_{TEM} (49.4%) and bla_{CTX-M} (3.5%). Pulsed-field gel electrophoresis (PFGE) applied to 45 representative *E. coli* isolates revealed that resistance to ceftiofur is spread both horizontally and clonally. In addition, the emergence of extended-spectrum β-lactamase-producing *E. coli* isolates carrying bla_{CTX-M} was observed in 2011 and 2012 in distinct clones. The most predominant plasmid incompatibility (Inc) groups were IncFIB, IncI1, IncA/C and IncFIC. Resistance to gentamicin, kanamycin and chloramphenicol as well as the frequency of bla_{TEM} and IncA/C significantly decreased over the study period, whereas the frequency of IncI1 and multidrug resistance to seven antimicrobial categories significantly increased. These findings reveal that extended-spectrum cephalosporin-resistant porcine *E. coli* isolates in Québec belong to several different clones with diverse antimicrobial resistance patterns and plasmids. Furthermore, bla_{CMY-2} was the major β-lactamase gene in these isolates. From 2011, we report the emergence of bla_{CTX-M} in distinct clones.

1. Introduction

The emergence and prevalence of extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* in food-producing animals are global public health concerns. Cephalosporins are used both in animals and humans to treat various bacterial infections. Ceftiofur, a third-generation cephalosporin, is used in pigs to treat respiratory diseases, lameness and enteric diseases, with the regulation of use varying from country to country [1]. Third-generation cephalosporins are considered to be a critically important class of antimicrobials as they are of very high importance to human medicine [2]. For example, the third-generation cephalosporin ceftriaxone is one of the drugs of choice to treat invasive paediatric salmonellosis [3]. Resistance to ceftiofur in food-producing animals is a potential public health issue because ceftiofur resistance determinants also confer cross-resistance to ceftriaxone and other third-generation cephalosporins. Ceftiofur has been used therapeutically in food-producing animals since 1989, and ceftiofur resistance mediated via the bla_{CMY-2} gene was first reported in 1998 [4]. Since 1997, the prevalence of ceftiofur resistance in *E. coli* isolates from clinically ill pigs has increased in Quebec, Canada, reaching ca. 22% in 2014 [5]. In Enterobacteriaceae, ESC resistance has been associated with the production of AmpC β-lactamases (e.g. CMY-2) and extended-spectrum β-lactamases (ESBLs) (e.g. CTX-M and some OXA enzymes) encoded by genes on transferable plasmids.

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Gram-negative bacteria producing the CTX-M and CMY families of β-lactamase are a major public health threat owing to the limited therapeutic options to treat infections with these bacteria as a result of co-association of resistance with that of other classes of antimicrobials, including aminoglycosides, sulphonamides, phenicols and tetracyclines, giving rise to multidrug-resistant (MDR) strains [6].

During the past decade, ESC resistance has been reported among clinical isolates of Enterobacteriaceae from humans and animals in Canada [7,8]. The objective of this study was to investigate the evolution with time of ceftiofur-resistant *E. coli* isolates from diseased pigs on different farms in Quebec, Canada, from 1997 to 2012 with respect to *E. coli* pathotypes, clones, plasmid types and antimicrobial resistance.

2. Materials and methods

2.1. Source of isolates

A total of 85 ceftiofur-resistant *E. coli* isolated from clinically diseased pigs from 1997 to 2012 were obtained from the strain collection of the OIE (World Organisation for Animal Health) Reference Laboratory for *Escherichia coli* (EcL) (Faculté de médecine vétérinaire de l'Université de Montréal, St-Hyacinthe, QC, Canada). Isolates from possible cases of *E. coli* infection from routine bacteriology diagnostic laboratories were submitted to the EcL for further analysis. Pathogenic isolates based on virulence gene determination were tested by the disk diffusion (Kirby–Bauer) assay for antimicrobial resistance, and those resistant to ceftiofur were selected for this study. The ceftiofur-resistant *E. coli* isolates were mainly recovered from pigs with diarrhoea, respiratory problems or oedema disease from 85 separate samples from 74 different cases on 41 distinct farms (Supplementary Table S1). For DNA preparation, individual colonies on MacConkey agar plates (Oxoid Company, Nepean, ON, Canada) were inoculated into 5 mL of Luria Bertani (Difco, Mississauga, ON, Canada) broth and were incubated overnight at 37 °C. DNA templates were prepared from *E. coli* isolates by boiled cell lysis for examination by PCR as described previously [9].

2.2. Detection of virulence genes and determination of pathotypes

Data for virulence genotyping were extracted from the EcL collection (1997–2007) and the APZEC database (2008–2012) (http://www.apzec.ca). Briefly, virulence gene typing was carried out at the EcL by PCR or colony hybridisation using radioactively labelled (^{32}P) DNA probes for virulence genes that define the *E. coli* pathotypes commonly found in pigs [enterotoxigenic *E. coli* (ETEC) (*eltB*, *estA* and *estB*); enteropathogenic *E. coli* (EPEC) (*eae*); Shiga toxin-producing *E. coli* (STEC) (*stxA* and *stx2A*); and extraintestinal pathogenic *E. coli* (ExPEC) (*cnf*, *papC*, *iucD* and *tsh*)] and for the genes *faeG*, *fedA*, *fanC*, *fasA*, *f41*, *paa*, *aidA* and *astA*, which permit the identification of extended pathovirotypes. PCR and colony hybridisation procedures for detection of these genes were performed according to the protocol of the EcL available on the APZEC website (http://apzec.ca/en/Protocols) and in Supplementary Table S2, and as described previously [10].

2.3. Antimicrobial susceptibility testing

E. coli isolates were examined for susceptibility to the same 15 antimicrobial agents examined in the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) surveillance programme in Canada [11] using the disk diffusion (Kirby–Bauer) assay as previously described [12]. *E. coli* isolates were considered MDR based on the definitions described by Magiorakos et al (non-susceptibility to at least one antimicrobial agent in three or more antimicrobial-defined categories) [13]. Furthermore, different levels of multidrug resistance were defined as follows: MDR4, non-susceptible to at least one agent in only four antimicrobial categories; MDR5, non-susceptible to at least one agent in only five antimicrobial categories, etc.

CTX-M-positive isolates were confirmed for ESBL production by the minimum inhibitory concentration method [14] using V2AGNF and ESB1F plates in the Sensititre system (Thermo Fisher, Oakwood Village, OH, USA).

2.4. β-Lactamase genotyping

Isolates were screened for the presence of the $bla_{\text{CMY-2}}$, bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV} and $bla_{\text{OXA-1}}$ genes by multiplex PCR as described previously [15,16]. Isolates that were PCR-positive for $bla_{\text{CTX-M}}$ were examined for the presence of $bla_{\text{CTX-M-15}}$ because of its importance in human medicine using the primers CTX-M -15-F (5′-CACACGTGGAATTTAGGGA-3′) and CTX-M-15-R (5′-GCCGTCTAAGGCGATAAAC-3′) as described previously [17].

2.5. Plasmid replicon typing and determination of class 1 and 2-integrons

Plasmid incompatibility (Inc) grouping was conducted using PCR replicon typing as described by Carattoli et al for 18 Inc groups [18]. A multiplex PCR assay targeting class 1 and 2 integrons (*int1* and *int2*) was performed to investigate the presence of integrons using previously described primers and conditions [19].

2.6. Phylogenetic grouping

Phylogenetic grouping was performed using a multiplex PCR-based assay as described by Clermont et al [20].

2.7. O serotyping

O serotyping was determined by standard agglutination methods [21] for 86 O serogroups associated with swine disease. O antisera were produced at the EcL according to standard methods [21].

2.8. Pulsed-field gel electrophoresis (PFGE)

E. coli isolates were analysed for genetic relationships by PFGE using the restriction enzyme *Xba*I as described by PulseNet [22]. PFGE patterns were analysed using BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated by the unweighted pair-group method using arithmetic averages (UPGMA), based on the Dice similarity coefficient (optimisation 1%; tolerance 1.2%).

2.9. Statistical analysis

Statistical analysis was carried out using JMP v.10 (SAS Institute, Cary, NC). The χ^2 test or Fisher's exact test, as appropriate, was used to compare the significance of differences in the prevalence of traits (genetic and phenotypic) with respect to different factors (e.g. study time, pathovirotypes). Odds ratios (ORs) and their 95% confidence intervals (CIs) were used to evaluate the associations

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